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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Purpose of this study: To test the hypothesis that aberrant chromatin modification plays a critical role in prostate cancer progression, we proposed to identify altered histone modification patterns linked to progression of prostate cancer and then identify the enzymatic activities that are responsible for the alteration.

Scope of this study: We proposed to analyze histone acetylation and phosphorylation patterns on androgen receptor (AR) target genes such as PSA and other genes important for cell proliferation in malignant prostate cancer cells at different stages as well as normal prostate epithelial cells. Comparison of the patterns of histone modifications between these cells will allow us to identify the chromatin modifications that are altered during the progression of prostate cancer. Identification of specific changes in histone modifications between normal and cancer cells will help identify the responsible enzymatic activities.

Major findings during the first year: We began our study by examining histone modification such as acetylation on AR target gene PSA in the androgen-dependent LNCaP cells and have made the following findings with the details incorporated in the attached manuscript:

1. We observed that DHT induced marked histone hyperacetylation at both the promoter and the enhancer of PSA gene.
2. We observed that DHT induced the recruitment/occupancy of nuclear receptor coactivators including members of the p160/SRC-1 family and CBP/p300. Both the p160 coactivators such as ACTR and SRC-1 and CBP/p300 proteins are demonstrated to possess histone acetylase (HAT) activities.
3. We demonstrated that elevation of ACTR protein level in the LNCaP cells increased DHT induction of PSA gene expression, suggesting the involvement of HAT protein ACTR in AR-mediated gene expression.
4. We also observed that anti-androgens such as casodex could induce the recruitment/occupancy of histone deacetylases such as HDAC1 and HDAC2. These proteins are components of the nuclear receptor corepressor complexes.

Significance: These findings indicate that histone acetylation is indeed involved in AR-mediated control of gene expression and that several of the known enzymatic activities (HAT and HDAC) that control the level of histone acetylation are likely responsible for the observed ligand-induced histone acetylation at PSA gene. These results will also help us set up the stages for the next phase of our study.

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Table of Contents

INTRODUCTION

Progression of prostate cancer (PCa) from hormone-dependent to hormone-refractory state represents one of the major hurdles in the successful treatment of cancer patients. Early studies showed that nearly all cancers retain androgen receptor (AR)-mediated signaling pathway. One of the working models has been that shifts from paracrine to autocrine expression of growth factors and abnormal function of their receptors contributes to the progression to androgen-independent cancer through modulating the function of AR. We proposed a new hypothesis that chromatin histone modification and remodeling could be a key step in PCa progression. The purpose of this study is to test this new hypothesis. We proposed to identify altered histone modification patterns linked to progression of prostate cancer and then identify the enzymatic activities that are responsible for the alteration. The cope of this study is to first analyze histone acetylation and phosphorylation patterns on androgen receptor (AR) target genes such as PSA and other genes important for cell proliferation in malignant prostate cancer cells at different stages as well as normal prostate epithelial cells. Comparison of the patterns of histone modifications between these cells will allow us to identify the chromatin modifications that are altered during the progression of prostate cancer. Identification of specific changes in histone modifications between normal and cancer cells will then help identify the responsible enzymatic activities.

RESEARCH ACCOMPLISHMENTS (June 2001-June2002)

Task 1. Identify abnormal histone acetylation and phosphorylation at important androgen receptor target genes and other genes associated with prostate cancer progression (months 1-18):

We began our study by examining histone modification such as acetylation on AR target gene PSA in the androgen-dependent LNCaP cells and have made the following significant findings with the details incorporated in the attached manuscript:

- We observed that DHT induced marked histone hyperacetylation at both the promoter and the enhancer of PSA gene (Figure 4).
- We observed that DHT induced the recruitment/occupancy of nuclear receptor coactivators including members of the p160/SRC-1 family and CBP/p300 (Figure 1). Both the p160 coactivators such as ACTR and SRC-1 and CBP/p300 proteins are demonstrated to possess histone acetylase (HAT) activities.
- We also observed that anti-androgens such as casodex could induce the recruitment/occupancy of histone deacetylases such as HDAC1 and HDAC2 (Figure 3). These proteins are components of the nuclear receptor corepressor complexes.

Significance: These findings indicate that histone acetylation is indeed involved in AR-mediated control of gene expression and that several of the known enzymatic activities (HAT and HDAC) that control the level of histone acetylation are likely responsible for the observed ligand-induced histone acetylation at PSA gene. These results also help us set up the stages for the next phase of our study to identify the difference in chromatin modification between normal and malignant prostate epithelial cells and between androgen-dependent and –independent prostate cancer cells.

To identify the difference in chromatin modification between normal and malignant prostate epithelial cells, we will perform similar experiments as with LNCaP cells but with normal prostate epithelial cells.

We will use an immortalized normal prostate epithelial cell line (RWPE-1) established by Dr. Mukta M. Webber at Michigan State University for this particular experiment. We are currently in the process of obtaining the cell line from Dr. Webber's laboratory.

To identify the difference in chromatin modification between androgen-dependent and –independent prostate cancer cells, we will use the LNCaP derivative cells as proposed in the grant application. We have already started the culture of C4-2 and C4-2B cells, which were established by Dr. Leland Chung's laboratory.

We have also tried several times to detect basal level or hormone induced histone phosphorylation at PSA and other target genes without a whole lot of success. We are currently examining whether the reagents we used, particularly the anti-phosphorylated H3 antibody from a commercial source, are of good quality for our assay. If not, we might have to generate the antibody ourselves. The other possibility is that histone phosphorylation may play a significant role in AR-mediated control of gene expression.

Task 2. Determine the role of specific histone acetyltransferases (HATs), histone deacetylases (HDACs) and the histone kinases in prostate cancer progression (months 18-36):

Although we proposed to take on Task 2 later in this study, we have recently made significant progress related to this task.

- We demonstrated that elevation of ACTR protein level in the LNCaP cells increased DHT induction of PSA gene expression, suggesting the involvement of HAT protein ACTR in AR-mediated gene expression (Figure 8).

As mentioned above and described in details in the attached manuscript, we have observed that DHT induced the recruitment/occupancy of nuclear receptor coactivators including members of the p160/SRC-1 family and CBP/p300 (Figure 1). Both the p160 coactivators such as ACTR and SRC-1 and CBP/p300 proteins are demonstrated to possess histone acetylase (HAT) activities. We have also observed that anti-androgens such as casodex could induce the recruitment/occupancy of histone deacetylases such as HDAC1 and HDAC2 (Figure 3). These proteins are components of the nuclear receptor corepressor complexes. Therefore, at the minimum, we already identified that the p160/SRC-1 HAT proteins and the CBP/p300 HAT proteins are involved in AR-mediated activation of gene expression in the presence of androgen DHT, and that histone deacetylases such as HDAC1 and HDAC2 are likely to play a role in AR-mediated repression of gene expression in the presence of anti-androgen Casodex.

We currently determine whether histone phosphorylation and its responsible kinases play a significant role in AR-mediated control of gene expression, as described above in Task 1.

REPORTABLE OUTCOMES:

a manuscript, Yang, H-Q., Louie M., Ma, A., Zou, J.X., Kung, H-J., and Chen, H-W. Androgen-dependent direct recruitment of RNA polymerase II and CTD kinases via AR-p160 coactivators. (submitted). 2002

CONCLUSIONS:

Our studies during the first year indicate that histone acetylation is indeed a mechanism in AR-mediated control of gene expression and that several of the known HAT proteins such as the p160 coactivators play important role in androgen induction of gene expression. The identification of p160 coactivators involved in control of AR target gene expression is of significance, particularly in light of the recent findings that members of the p160 coactivator family are overexpressed in malignant prostate tissues from PCa patients (Gregory et al. 2001). Since the functions of HATs and HDACs are amenable to enzymatic inhibitions (Chen et al. 2001), one of the implications of our recent findings is that histone modification enzymes could represent novel legitimate targets for drug development to battle prostate cancer.

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Gregory, C.W., He, B., Johnson, R.T., Ford, O.H., Mohler, J.L., French, F.S., and Wilson, E.M. 2001. A mechanism for androgen receptor-mediated prostate cancer recurrence after androgen deprivation therapy. *Cancer Res* **61**: 4315-9.

APPENDICES:

a manuscript, Yang, H-Q., Louie M., Ma, A., Zou, J.X., Kung, H-J., and Chen, H-W. Androgen-dependent direct recruitment of RNA polymerase II and CTD kinases via AR-p160 coactivators. (submitted). 2002

Androgen-dependent direct recruitment of RNA polymerase II and CTD kinases via androgen receptor-p160 coactivator complex

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Abstract

Nuclear receptors and their cognate ligands activate target genes by binding to hormone-responsive enhancers. The tissue-specific expression of PSA, a prostate cancer marker, is regulated by an androgen-responsive distant enhancer. Here we show that androgen induces robust recruitment of the androgen receptor (AR), p160/SRC-1 and CBP/p300 coactivators specifically at the PSA enhancer, and simultaneously diminishes AR-independent occupancy of histone deacetylases at both the promoter and enhancer. These combined events lead to targeted histone hyperacetylation and transcriptional activation. Unexpectedly, we found that RNA polymerase II (Pol II) with its carboxy-terminal domain (CTD) distinctively phosphorylated is directly recruited at the enhancer in a hormone-dependent manner and that the isolated PSA enhancer can mediate efficient androgen induction of transcription independent of the proximal promoter. In search of the mechanism whereby Pol II at the enhancer acts upon the promoter, we identified the co-occupancy of CTD kinases Cdk7 and Cdk9/P-TEFb with Pol II. Strikingly, inhibition of the CTD kinase activity with flavopiridol blocks Pol II 'tracking' from the enhancer to the promoter and selectively abolishes androgen induction of PSA expression. In addition, we demonstrate that elevated levels of p160 coactivator ACTR directly facilitate Pol II recruitment to the enhancer. These results support a model in which nuclear receptors and their cofactors mediate hormone induction by serving as a staging platform for Pol II recruitment and initiation.

Introduction

Androgen is a key regulator of cell growth and differentiation in male sexual development and function, as well as the progression of prostate cancer. Androgen deprivation therapy results in clinical regression of prostate tumors and decreased serum levels of PSA (prostate-specific antigen), a well-known marker of prostate cancer. These hormonal effects are mediated by the androgen receptor (AR), a member of the nuclear receptor super-family which are ligand-dependent transcription factors. Upon binding to hormone, AR, like other steroid receptors, dissociates from heat-shock proteins and translocates to the nucleus, where it regulates transcription by association with the androgen response elements (AREs) in the regulatory region of target genes (for reviews, see Mangelsdorf et al. 1995).

Recently, a growing number of nuclear proteins have been found to associate with nuclear receptors, including AR, and are postulated to mediate transcriptional control (for reviews, see Glass and Rosenfeld 2000; McKenna and O'Malley 2002). One group of such cofactors is the p160 coactivator family, including SRC-1, TIF2/GRIP1, and ACTR/AIB1/RAC3/TRAM1/pCIP. Several functional domains are conserved among the p160 family members, including the central receptor interaction domain (RID) and a C-terminal domain responsible for the cooperative interaction with CBP/p300. The p160

coactivators associate with nuclear receptors in a hormone-dependent fashion primarily through the central RID that harbors several LXXLL motifs. In the case of AR, strong ligand-independent interactions between the C-terminus of p160 coactivators and the N-terminal transactivation domain of AR were also observed in in vitro protein-protein interaction assays (Alen et al. 1999; Bevan et al. 1999; Ma et al. 1999). These results raise the possibility that p160 coactivators may function in both an androgen-dependent and -independent manner.

While p160 coactivators possess intrinsic histone acetylase (HAT) activities, they are also capable of recruiting other HAT proteins such as CBP, p300 and PCAF (Chen et al. 1997; Spencer et al. 1997). In addition, p160 coactivators associate with the nuclear protein arginine methyltransferases CARM1 and PRMT (Chen et al. 1999a; Ma et al. 2001; Xu et al. 2001). Both the nuclear acetylases and methylases can regulate transcription by modifying both nucleosomal and non-nucleosomal nuclear proteins (Chen et al. 1999b; Chen et al. 2001; Xu et al. 2001). Consistent with the notion that nucleosomal histones may be the primary targets of cofactor regulation, histone deacetylases (HDACs) were found in transcriptional repressor complexes which associate with unliganded nuclear receptors such as retinoic acid receptors (RARs) and thyroid hormone receptors (TRs)(Ordentlich et al. 2001; Privalsky 2001). These observations suggest that hormone controls gene expression by acting as a switch to regulate the

assembly at chromatin of cofactor complexes with opposing enzymatic activities to remodel the chromatin structure.

How the p160 coactivators promote RNA polymerase II (Pol II) transcription is poorly understood. Domain mapping experiments based on reporter assays suggest that the transcriptional activation activity of p160 coactivators is largely attributable to its recruitment of CBP/p300 and its associated HAT activities (Chen et al. 1997; Torchia et al. 1997; Kraus et al. 1999; Liu et al. 2001). In contrast, substantially earlier experiments indicate that CBP/p300 may interact directly with the Pol II complex (Nakajima et al. 1997; Cho et al. 1998). This idea lost currency with the discovery of the importance of the HAT activity in transcriptional activation and the more recent demonstration that the simple recruitment and assembly of Pol II machinery in mammalian cells is not sufficient for productive transcription (Dorris and Struhl 2000; reviewed in Lemon and Tjian 2000). In this regard, the dynamic phosphorylation of Pol II carboxy terminal domain (CTD) has been strongly implicated in different stages of PolII function, from transcriptional initiation to mRNA processing (reviewed in Orphanides and Reinberg 2002). Sequential phosphorylation of CTD at serine 5 and 2 of the heptad repeat correlates with the induction of transcription (Nissen and Yamamoto 2000; Cho et al. 2001). Three cyclin-dependent kinases (Cdk) were shown to directly phosphorylate CTD. Both Cdk7/cyclin H and Cdk8/cyclin C are believed to phosphorylate the CTD after the formation of pre-

initiation complex. P-TEFb (positive-transcription elongation factor b), consisting of Cdk9 and primarily cyclin T, has been shown to activate HIV transcription by facilitating the release of stalled PolII from arrest near the promoter (Garber and Jones 1999; Price 2000). Interestingly, P-TEFb was recently shown to directly associate with PolII CTD and could potentially mediate the function of enhancers (Taube et al. 2002).

Nuclear receptor-dependent transcriptional activation is mediated by hormone response elements such as the androgen-controlled enhancer at approximately –4.2 kb on the PSA gene. High-level expression of PSA is restricted to the prostate and appears to be dependent on androgen induction. Such cell type-specific androgen response is conferred by a region approximately –4,800 to –3300 bp from the transcription initiation site; the minimal core required for androgen induction of transcription is a fragment of less than 500 bp encompassing a prominent androgen-inducible DNase I hypersensitive site (Schuur et al. 1996; Cleutjens et al. 1997b). Mutational analysis demonstrates multiple tandem androgen response elements (AREs) in the core region with each contributing to synergistic AR binding and activation (Huang et al. 1999; Farmer et al. 2001). Interestingly, two additional AREs (ARE I at –170 and ARE II at -400) were identified in the PSA proximal promoter region. However, little androgen-induced transcriptional activity was observed in cultured prostate cells or transgenic animals carrying only the proximal promoter (Schuur et al. 1996; Cleutjens et al. 1997a; Cleutjens et al. 1997b),

despite the fact that both AREs form stable AR complexes in vitro (Cleutjens et al. 1996; Sun et al. 1997).

Previously, we and others demonstrated that hormone induces histone hyperacetylation at the proximal promoters of nuclear receptor target genes via the recruitment of coactivator complexes (Chen et al. 1999b; Shang et al. 2000). To further understand the molecular underpinnings of histone acetylation in transcriptional regulation and investigate the mechanism of hormone induction of gene expression through distant enhancers, we analyzed androgen-induced recruitment of AR and its associated coactivators to the entire 5' regulatory region of the PSA gene in hormone responsive LNCaP cells. Here, we show that upon androgen stimulation, p160 and CBP/p300 coactivators are preferentially recruited to the enhancer. This observation has led to our unexpected finding that RNA polymerase II is directly recruited to the enhancer in an androgen-dependent manner and that the Pol II CTD kinase activities are critical for the tracking of Pol II from enhancer to promoter.

Results

AR and p160-CBP/p300 coactivators are preferentially recruited to the PSA enhancer upon androgen stimulation

Previous studies demonstrated that a 6-kb fragment containing the promoter and the upstream sequence of the PSA gene is sufficient to direct androgen induction of reporter gene expression in the prostate of transgenic animals and in the human prostate cancer cell line LNCaP (Schuur et al. 1996; Cleutjens et al. 1997a; Cleutjens et al. 1997b). In order to investigate the role of AR and its associated coactivators in PSA induction, we first analyzed androgen-induced AR and coactivator occupancy over the entire 6-kb upstream regulatory region of PSA by chromatin immunoprecipitation (ChIP) assay using LNCaP cells. For semi-quantitative measurement of co-immunoprecipitated chromatin DNA, we used a panel of PCR primer pairs with approximately equal efficiency to amplify partially overlapping fragments (~300 to 500 bp) encompassing the entire 6-kb regulatory region (Fig. 1A, panel a and b). The enhancer sequence was amplified as fragments D, E and F, while the sequence of proximal AREs and initiation site was covered by fragments O and P. The fragment E contains the high-affinity ARE found at the center of the enhancer.

As shown in Figure 1A (panel c), ChIP assay with anti-AR antibody demonstrated clearly that, upon androgen (dihydrotestosterone, DHT) induction, AR was strongly recruited to the enhancer region and this AR occupancy persisted hours after DHT addition. Interestingly, the association of AR with the proximal promoter sequence was only marginal after DHT stimulation. Likewise, no significant AR occupancy was seen at the other regions of the 6-kb PSA upstream sequence. To determine whether the p160 coactivators are involved in the androgen induction of PSA gene expression, we also measured the recruitment of p160 coactivators and their associated transcriptional coregulators such as CBP and p300. Indeed, robust recruitment of ACTR, TIF2 and CBP to the PSA 5' regulatory region was observed when cells were treated with DHT (Fig. 1A, panels d, e and f). A similar pattern was obtained with the p300 specific antibody (data not shown). Importantly, as seen for AR, androgen-induced coactivator recruitment was highly restricted to the enhancer, as only a slight increase in proximal promoter occupancy could be observed with TIF2 and CBP. To determine whether the differential recruitment at enhancer and promoter elements of AR and its coactivators was reflected in their ability to mediate androgen induction of transcription, we transfected LNCaP cells with two reporters containing either the 6 kb upstream sequence or the 630-bp proximal sequence of PSA. Results in Figure 1B indicate that it is the enhancer-containing 6-kb upstream sequence, not the proximal promoter sequence, that was capable of mediating androgen induction. In agreement with the ChIP results, co-

transfection of coactivator ACTR and TIF2 constructs further potentiated androgen-stimulated transcription through the 6-kb sequence, but not the 630-bp promoter (Fig. 1B, panels a & b respectively). Taken together, these results suggest strongly that the androgen-responsive enhancer of the PSA gene mediates transcriptional activation through direct recruitment of AR and the p160-CBP/p300 coactivator complex. The inability of the proximal AREs to mediate androgen-induced transactivation is attributed to their lack of strong activity to recruit AR and its coactivators in androgen responsive cells.

Androgen induces histone hyperacetylation at both the enhancer and proximal promoter

Having demonstrated that androgen-induced recruitment of coactivators is highly localized to the upstream enhancer, we examined whether the HAT activity associated with these coactivators also targeted this region. As expected from the strong occupancy of HAT coactivators at the enhancer, two hours of DHT stimulation resulted in a marked increase (up to 6 fold) in histone H4 acetylation in the enhancer region (Fig. 2). Surprisingly, a strong androgen-induced histone hyperacetylation at the proximal promoter region covered by fragments O (3 fold) and P (5 fold) was also detected. Moreover, the region between enhancer and promoter displayed significant androgen-

independent histone H4 acetylation, which could be markedly enhanced by androgen stimulation. In contrast, regions that are ~2- or 4-kb upstream of the enhancer, covered by fragments A, B, U1 or U2 respectively, showed minimal basal level and hormone-induced histone acetylation (Fig. 2). These results suggest that androgen induces histone hyperacetylation over a broad region of PSA regulatory sequence from the enhancer to the promoter. Our results also imply that the nucleosomes of this region are acetylated distinctively as sub-domains both in the presence and absence of androgen, with a potential boundary established upstream of the 6-kb regulatory sequence.

HDACs are recruited to the enhancer and proximal promoter to suppress PSA expression by anti-androgen

The relative levels of histone acetylation in vivo at a given location of chromatin are likely determined by the dynamic action of both HATs and HDACs. Since PSA enhancer and promoter regions appear to have histones hypoacetylated more than other regions in the absence of hormone, we sought to investigate whether HDACs occupy the two sub-domains of PSA chromatin. Since HDAC1 and HDAC2 are highly expressed in most cells including LNCaP (data not shown), we examined their association with PSA enhancer and promoter by ChIP assay with specific anti-HDAC antibodies. Remarkably, as shown in Fig. 3A, in the absence of hormone, we detected strong occupancy of

HDAC1 at both the enhancer and proximal promoter regions. Consistent with the notion that, in the absence of hormone, AR is localized in the cytoplasm, we could not detect significant AR occupancy at the PSA gene, as shown in this and the previous experiment (Fig. 1A). We therefore concluded that transcription factor(s) other than AR likely mediate the HDAC1 occupancy. Strikingly, this AR-independent HDAC1 recruitment is diminished upon androgen (DHT) stimulation. In contrast, exposure of cells to the AR antagonist Casodex (bicalutamide) resulted in significantly increased recruitment of HDAC1 and the induction of HDAC2 recruitment at both enhancer and promoter (Fig. 3A). Interestingly, anti-AR antibody detected the cross-linking of AR to both enhancer (fragments E and F) and proximal promoter (fragments O and P), indicating that, like DHT, anti-androgen Casodex can trigger AR nuclear translocation and its association with AREs at AR target genes in LNCaP cells.

To assess the role of HDACs in control of PSA promoter activity, we transfected LNCaP cells maintained in hormone-depleted media with the two PSA reporter constructs, and treated the cells with HDAC inhibitor trichostatin A (TSA) either alone or in combination with DHT. In contrast to the low activity of DHT on the PSA proximal promoter, TSA alone at 100 nM was able to significantly induce its transcription; and a synergistic effect was observed when cells were treated with both TSA and DHT (Fig.

3B). Similar effects were seen with the 6-kb versionof the reporter containing both the proximal promoter and enhancer.

These results suggest that, in the absence of hormone, PSA expression is inhibited by the activity of HDACs, which occupy the PSA regulatory sequence in an AR-independent manner. Upon androgen induction, the recruitment of AR and the p160-CBP/p300 coactivators invokes the dislodging of HDACs from their local chromatin occupancy, therefore facilitating histone hyperacetylation at both the promoter and enhancer. Our finding that Casodex induces HDAC recruitment argues that AR antagonists inhibit activation by promoting histone deacetylation.

RNA Polymerase II is directly recruited at PSA enhancer upon androgen induction

Transcription factors and their coregulators are believed to regulate transcription, in part, by controlling the recruitment and/or assembly of the general transcription machinery (Lemon and Tjian 2000). We, therefore, asked whether RNA polymerase II (Pol II) is recruited to the upstream sequence of the PSA gene in response to androgen stimulation. In agreement with our previous results that hormones induce Pol II recruitment to the promoters of estrogen receptor (ER) target genes (Chen et al. 1999b), significant recruitment of Pol II to the PSA promoter region is observed upon androgen induction

(Fig. 4A, open bars). We showed earlier in Figure 1 that hormone induced coactivator recruitment is targeted primarily to the enhancer region of PSA. To learn whether androgen-induced Pol II recruitment is targeted similarly, we first analyzed the same anti-Pol II co-precipitated DNA for any potential Pol II cross-linking over the enhancer region. Intriguingly, strong androgen-dependent Pol II recruitment to the enhancer (Fig. 4A, filled bars) was also detected. Judging by the signal intensity, the DHT-induced Pol II cross-linking at the enhancer is more abundant than that at the promoter. This unexpected result prompted us to extend this analysis to the other region of the PSA regulatory sequence. As shown in Figure 4B, remarkably, a significant level of Pol II cross-linking was observed over the region between enhancer and promoter. However, when regions further upstream were analyzed, little Pol II occupancy was detected (data not shown).

An attractive model of how an enhancer influences the function of the transcription machinery involves the physical interactions of protein complexes assembled at the enhancer with those at the promoter, leaving a DNA loop in between (i. e. the looping model). Formally, the cross-linking of Pol II to the enhancer can be attributed either by the contact of protein complexes formed at the enhancer with Pol II assembled at the promoter, or by the occupancy of Pol II at the enhancer independent of promoter (Fig. 4C). We reasoned that if the first scenario is true, we should be able to detect efficient

cross-linking of AR and p160-CBP/p300 complex to the promoter sequence, a result inconsistent with that shown in Figure 1. To further discern the two possibilities, we took advantage of our observation that AR is primarily recruited to enhancer. If Pol II is directly recruited to the enhancer, it would physically co-occupy the enhancer with AR. Therefore, fragmented chromatin-protein adducts, immuno-precipitated by anti-Pol II antibody, should contain the enhancer chromatin fragment that is also cross-linked with AR. As shown in Figure 4D, panel a, when the eluate of anti-Pol II precipitates were re-precipitated with anti-AR antibody, indeed, the second precipitates contained the enhancer DNA. In contrast, no significant amount of promoter sequence can be detected, in accordance with the low occupancy of AR at promoter. When the same eluate of Pol II precipitates was re-precipitated with anti-Pol II antibody, strong hormone-dependent cross-linkings were seen at both enhancer and promoter, indicating that the failure to detect promoter sequence in the anti-AR re-IP is not due to the preferential loss of promoter chromatin fragment during the elution. Again, consistent with the notion that AR and Pol II co-occupy enhancer but not promoter, a sequence from the enhancer but not promoter was detected after the anti-AR precipitates were subsequently immunoprecipitated with the Pol II antibody (Fig. 4D, panel b). These results indicate that, upon androgen stimulation, Pol II is directly recruited to the enhancer, independent of the PSA promoter.

Pol II recruited at the enhancer and promoter is differentially phosphorylated at CTD

Having shown that the Pol II proteins at the enhancer and promoter of PSA are two molecular entities, we began to examine their functional status. Phosphorylation of the carboxy-terminal domain (CTD) of Pol II has been recognized as a hallmark of Pol II functional transition from its promoter-entry state to a transcriptionally engaging mode (Dahmus 1996). Pol II CTD in mammalian cells contains more than 50 tandem repeats of YSPTSPS consensus sequence, with ser 2 and 5 predominantly phosphorylated in vivo. During the formation of the transcription complex or shortly after initiation of transcription, the CTD is phosphorylated on ser 5. The escape of Pol II from arrest and its full engagement in elongation is often marked by CTD ser 2 phosphorylation (Orphanides and Reinberg 2002). We performed ChIP assay with Pol II monoclonal antibodies H5 and H14, which recognize CTD phosphorylated at ser 2 and 5 respectively (Patturajan et al. 1998). The results showed that, within 10 min of androgen stimulation, ser 5-phosphorylated Pol II was strongly cross-linked to the enhancer (E fragment). In contrast, ser 2-phosphorylated Pol II did not appear to strongly occupy the enhancer, but it was associated with the promoter (Fig. 5). These data suggest that Pol II recruited at the enhancer is phosphorylated primarily at CTD ser 5, while PolII associated with the

promoter is increasingly phosphorylated at ser 2 and ser 5 during the early phase of hormone stimulation.

To assess the extent of CTD phosphorylation, we performed ChIP assay with 8WG16 antibody, which recognizes both unphosphorylated and partially phosphorylated CTD. During the course of one hour of androgen stimulation, increasing amounts of Pol II recognized by 8WG16 was cross-linked to the entire PSA gene locus. During the same time, Pol II was increasingly phosphorylated at the promoter and transcribed regions (fragments S, W and Z, Fig. 5). Our findings suggest that the majority of the Pol II recruited at the PSA gene locus is likely partially phosphorylated, echoing a recent, similar observation made in the yeast system (Komarnitsky et al. 2000).

The isolated enhancer mediates strong androgen induction of transcription independent of PSA proximal promoter

Our observation that Pol II occupying the PSA enhancer is phosphorylated at its CTD suggests that Pol II might function to initiate transcription from the enhancer. To test this, we made promoter-less reporter constructs where fragments of PSA 5' upstream sequences containing the enhancer were directly linked to the luciferase gene. Remarkably, when transfected into LNCaP cells, the reporter linked to a PSA 5' fragment

containing essentially the core enhancer sequence (PSA-E 1.5 kb) mediated a robust DHT-induced reporter gene activity similar to the reporter construct that contains both the enhancer and promoter (PSA 5.8 kb; Fig. 6). Surprisingly, however, a similar reporter construct containing 500 bp extra sequences downstream of the enhancer (PSA-E 2.0 kb) was completely inactive in response to DHT induction, suggesting that at least part of the enhancer function is controlled by an enhancer-context dependent mechanism. By Northern analysis with the 1.5 kb enhancer sequence as a probe, we were unable to detect any mature transcripts initiated around the enhancer region (data not shown), indicating that the enhancer does not serve as a gene promoter. Nevertheless, these results indicate that the androgen responsive enhancer of the PSA gene not only can independently recruit Pol II, but also has the potential to mediate transcriptional initiation.

CTD kinase(s) are involved in mediating Pol II tracking from enhancer to promoter

The observation that the Pol II recruited at the enhancer is phosphorylated at ser 5 but that at the promoter is phosphorylated at both ser 2 and ser 5 of the CTD heptad suggests to us that CTD phosphorylation may be important for Pol II recruited at the enhancer to act on the promoter. The three CTD kinases (Cdk7, Cdk8 and Cdk9), displaying distinct substrate preferences in vitro have been implicated in the control of Pol II activities. Cdk7 may phosphorylate CTD on ser 5 right before the initiation of transcription while

Cdk9/P-TEFb may phosphorylate ser 2 afterwards (Cho et al. 2001; Orphanides and Reinberg 2002). The CTD kinases exist in different subassemblies of protein complexes and are not the obligatory components of the general factors in the machinery (Rachez and Freedman 2001). Nevertheless, their co-localization with hypophosphorylated form of PolII at gene loci is considered as an indicative of their functional involvement in the regulation of specific gene expression (Lis et al. 2000; Barboric et al. 2001). As a first step to determine whether the CTD kinases play a role in hormone induction of transcription, we examined their recruitment at the PSA gene before and after androgen treatment. As shown in Figure 7A, lanes 1-3 and 6-8, Cdk7, Cdk8 and Cdk9 are differentially recruited to the PSA enhancer and promoter. Thus, while Cdk9 occupies both the promoter and the enhancer in the presence of androgen, Cdk7 is likely recruited directly at the enhancer since much higher signal intensities of Cdk7 cross-linking was seen with enhancer than the promoter. Interestingly, Cdk8 was found to occupy the promoter and enhancer primarily in the absence of hormone. These results indicate that the Pol II CTD kinases Cdk7 and Cdk9/P-TEFb are likely involved in hormone induction of PSA transcription. Their co-occupancy with Pol II at the enhancer implies Cdk7 and/or Cdk9/P-TEFb might play a role in the functional transfer of Pol II from enhancer to promoter.

To determine if the CTD kinase activities of Cdk7 and Cdk9 are indeed required for Pol II function at the PSA gene, we resorted to the CTD kinases inhibitor flavopiridol. Flavopiridol is a Cdk inhibitor and has been shown to potently inhibit Cdk 8 and Cdk9/P-TEFb with an IC_{50} around 10 nM (Rickert et al. 1999; Chao et al. 2000; Chao and Price 2001). We first examined whether flavopiridol treatment affects the recruitment/occupancy of the three Cdks. As shown in Figure 7A, lanes 4, 5, and 9, 10, treating LNCaP cells with flavopiridol strongly inhibits androgen-induced occupancy of Cdk7, while the recruitment of Cdk9 is less significantly affected. Intriguingly, one-hour treatment with flavopiridol induces the recruitment of Cdk8 at the promoter and enhancer. These results further strengthen the notion that the kinase activities of Cdk7 and Cdk9 are important in Pol II function at the PSA gene.

Next, we analyzed the effect of flavopiridol on androgen-induced PSA expression by Pol II. Low concentration (< 300 nM) of flavopiridol does not show inhibitory effect on most gene transcription (Chao and Price 2001). RT-PCR results in Fig. 7B indicate that, when LNCaP cells were treated with 100 nM flavopiridol, DHT-induced PSA transcription was completely blocked. Consistent with the previous report, expression of AR itself and cell cycle genes such as Cdk 4 and cyclin D was not significantly affected. Finally, we examined the effect of flavopiridol treatment on androgen-induced Pol II recruitment/occupancy at PSA gene (Fig. 7C). Strikingly, treating cells simultaneously

with DHT and flavopiridol strongly blocked androgen-induced Pol II occupancy at the promoter (lanes 9 and 10) but enhanced that at the enhancer region (lane5). This effect was not seen at the promoter of other genes whose expression was not affected by the low concentration of flavopiridol (Fig. 7D). Since blocking transcriptional initiation or elongation would leave more Pol II assembled and stalled at the promoter, as demonstrated recently for the IL-8 gene (Barboric et al. 2001), the decrease in Pol II occupancy of the PSA promoter can not be attributed to the blockade of transcription initiation or elongation at the promoter. Our finding that decreased Pol II occupancy at promoter is accompanied by its simultaneously increased presence at enhancer suggests that Pol II recruited at the enhancer of PSA 'tracks' to the promoter and that flavopiridol perturbs this process by inhibiting the kinase activity of Cdk7 and/or Cdk9.

ACTR mediates androgen induction of PSA through facilitating Pol II recruitment at enhancer

Since p160 coactivators ACTR and TIF2 are recruited primarily at the enhancer of PSA (Fig. 1), we decided to test the hypothesis that p160 coactivators might play a role in androgen-induced Pol II recruitment at the enhancer. To this end, we ectopically expressed wild type and an amino-terminal truncated ACTR (A38) in LNCaP cells (Fig. 8A) and examined the effect of elevated expression of ACTR on androgen-induced PSA

expression and Pol II recruitment. As shown in Figure 8B, when LNCaP cells were stimulated with a sub-optimal concentration of DHT (1 nM), increasing the level of ACTR significantly enhanced androgen induction of PSA expression. Interestingly, ACTR-A38, which lacks the N-terminal 457 amino acids, showed much higher activity than the full-length ACTR, correlating with their different expression levels. Importantly, when Pol II recruitment was analyzed by ChIP assay, a marked increase in Pol II recruitment was detected at the enhancer but not the promoter, in cells ectopically expressing ACTR (Fig. 8C). To assess whether ACTR is in complex with Pol II in solution, we performed conventional co-immunoprecipitation assay (Fig. 8D). Indeed, we found that a sub-fraction of Pol II was in association with ACTR in solution. Interestingly, while the association of ACTR with AR is largely androgen dependent, its association with Pol II and CBP is hormone-independent, suggesting that ACTR might bring CBP and/or Pol II to the enhancer in pre-formed complexes. We also detected the association of ACTR with cyclin T1, a component of Cdk9/P-TEFb, indicating the possibility that P-TEFb might be recruited at enhancer through ACTR. Taken together, these results provide evidence that p160 coactivators such as ACTR are involved in androgen induction of PSA expression by facilitating Pol II recruitment to the enhancer.

Discussion

Transcriptional regulation by nuclear receptors presents a paradigm for understanding the molecular mechanism of coregulator function in cellular coordination of gene expression in response to hormone. In this work, we demonstrated that hormones such as androgen induce the assembly of the p160-CBP/p300 coactivator complex and the recruitment of RNA polymerase II directly to an androgen-controlled distant enhancer. We further showed that Pol II CTD kinases Cdk7 and Cdk9/P-TEFb are also recruited to the enhancer, which likely play a role in mediating Pol II "tracking" from enhancer to promoter.

Direct recruitment of Pol II at the distal enhancer as a mechanism of enhancer function

Enhancers are discrete DNA elements that mediate the transcriptional activation activities of DNA binding transcription factors at a distance from the promoter. One of the best characterized mammalian enhancers is the one from the interferon-β gene, where a group of transcription factors including the architectural chromatin protein HMGI(Y) bind cooperatively to the enhancer to form a complex called enhancersome (Merika and Thanos 2001). Recently, strong evidence was presented to support a model that HATs

and SWI/SNF complexes are sequentially recruited to the enhancer to remodel the local chromatin, followed by the recruitment of CBP-Pol II holoenzyme complex at the promoter (Agalioti et al. 2000). Since the interferon-β enhancer is located nearby to the promoter (less than 150 bp apart), it was not possible to discern whether Pol II was recruited directly at the enhancer or the promoter. In this regard, the positioning of a hormone-responsive enhancer over 4 kb upstream of the PSA promoter greatly aided our dissection of enhancer function. By analyzing systematically the occupancy of Pol II as well as AR and its coactivators over the entire PSA regulatory region, we found that androgen induces the recruitment of Pol II preferentially to the enhancer. We then demonstrated that AR and Pol II co-occupy strongly at the enhancer but not at the proximal promoter, thereby defying the explanation by the looping model in which the enhancer indirectly contacts Pol II complex assembled at the promoter by looping out the interval DNA. With highly focused analysis, Shang et al. recently reported the detection of androgen-induced Pol II association with both PSA promoter and enhancer (Shang et al. 2002). Although their study did not address the molecular nature of such association, their result is consistent with our conclusion that Pol II is directly recruited at the enhancer. More strikingly, we observed that the PSA enhancer region, in isolation, could mediate androgen induction of transcription, independent of the PSA promoter. Further, the CTD kinase inhibitor flavopiridol promoted Pol II occupancy at the enhancer, with a concomitant block of Pol II occupancy at the promoter. These data suggest that the

recruitment of Pol II at the enhancer and the promoter may be controlled by distinct mechanisms.

It has long been speculated that enhancers may function as an entry site for RNA polymerase to relay the transcriptional activation signals impinged at the enhancer to the nearby promoter (Muller and Schaffner 1990). The detection of Pol II-dependent transcripts within the locus control region (LCR) of β-globin genes, a unique example of enhancer-type elements, led to the notion that Pol II recruitment at the LCR may play an important role in perturbing the chromatin structure between LCR and promoter, thereby setting a transcriptional competent state for the entire locus (Gribnau et al. 2000). Recently, using ChIP assay, it was demonstrated that Pol II occupancy at LCR is controlled by an unidentified mechanism that may differ from that at the β-globin promoter (Johnson et al. 2001). Our finding that Pol II is recruited at a conventional enhancer, upon hormone induction, suggests that direct Pol II recruitment may represent a general mechanism underlying enhancer function.

How Pol II is recruited to the enhancer is currently unclear. Our results tend to support the assertion that Pol II is recruited to the enhancer through the p160-CBP/p300 coactivator complex. Strong biochemical evidence suggests that CBP/p300 either directly contacts Pol II or exists in a complex containing Pol II. Our co-immunoprecipitation

results indicated that a large proportion of CBP/p300 formed a complex with p160 coactivators such as ACTR and that at least a fraction of Pol II could be in association with ACTR. We also observed that treating LNCaP cells with the histone deacetylase inhibitor TSA could recapitulate, to some extent, hormone-mediated recruitment of Pol II (data not shown). These results are consistent with the hypothesis that the acetylase activity of p160-CBP/p300 is involved in the recruitment.

P-TEFb as a potential mediator for PolII "tracking" from enhancer to promoter

Conceptually, transcription factors/coactivators or Pol II recruited at a distant enhancer can act on the promoter by either the looping mechanism or tracking along the chromatin (Blackwood and Kadonaga 1998). Enhancers in mammalian cells are often more than 5 or 10 kb away from their cognate promoters; therefore, simply looping out the interval DNA to make the protein-protein contact between enhancer and promoter is less likely. We tend to favor the "facilitated tracking" mechanism for the Pol II complex to interact with the promoter, based opon the observations made in this study. Thus, we found that androgen-induced Pol II cross-linking appears to trail over the entire region of 6-kb PSA upstream sequence. Intriguingly, the pattern of androgen-induced histone hyperacetylation resembles the pattern of Pol II cross-linking. It is conceivable that the hyperacetylation of nucleosomal histones might help the transient association of Pol II

with chromatin elements downstream the enhancer, or that the "tracking" of Pol II complex perturbs the local chromatin structure through the chromatin remodeling activities that the Pol II might associate with. This model does not exclude the possibility that, during Pol II "tracking", interactions between Pol II complex or the complexes at the enhancer, and the general transcription factors or other complexes assembled at the proximal promoter, become firmly established, facilitating the functional transfer of Pol II to the promoter.

The consideration that Cdk7 and Cdk9/P-TEFb might be involved in Pol II "tracking" stems from our observation that these kinases cross-link to the enhancer. This notion is further supported by the finding that their kinase activities are required for the "tracking" of Pol II from enhancer to promoter. Between the two kinases, Cdk9/P-TEFb might be more directly involved in Pol II tracking than Cdk7. Cdk9/P-TEFb has substrate preference on ser 2 of Pol II CTD heptad, while Cdk7 may phosphorylate primarily ser 5. P-TEFb may also directly associate with PolII CTD through its component cyclin T. Interestingly, by tethering to DNA through Gal4 DBD P-TEFb was recently shown to activate transcription from sites either upstream or downstream of target genes (Taube et al. 2002). In this regard, our finding that P-TEFb is recruited, in a hormone-dependent manner, to the distant enhancer of PSA provide a physiologically relevant scenario where P-TEFb might function as a mediator of enhancer function.

p160 coactivators and androgen-independence of prostate cancer

Prostate cancer almost inevitably progresses from a hormone-dependent to a hormone-refractory state. PSA levels in blood correlate very closely with clinical states of the disease and has been considered as one of the most valuable tumor markers for cancer (Oesterling 1991). The underlying mechanisms of hormone independence in prostate cancer are still poorly understood. Elevated levels of PSA are commonly associated with the recurrence of the disease, and androgen receptor (AR) is strongly implicated in the progression. Therefore, a thorough understanding of the mechanism that governs the expression of PSA will shed light on the mechanism underlying the androgen independence of prostate cancer. Recently, aberrant expression of p160 coactivators was identified in multiple human malignancies including prostate cancer (Gregory et al. 2001). In this study, we observed that elevated level of ACTR increased PSA expression in the absence of hormone or at sub-optimal level of androgen. It would be of interest to study the involvement of p160 coactivators in the development of prostate cancer androgen-independence and its underlying mechanisms.

Materials and methods

Chromatin immunoprecipitation (ChIP) assay

LNCaP cells were grown to 70-90% confluence in RPMI 1640 (Gibco BRL) supplemented with 5% charcoal-dextran-stripped fetal bovine serum for at least 3 days prior to treatment with ligands. Cells were then fixed with 1% formaldehyde at room temperature for 10 minutes and washed with ice-cold PBS. After cells were scraped off in Buffer I (0.25% Triton X-100, 10mM EDTA, 0.5mM EGTA, 10mM HEPES, pH 6.5). Cell pellets were collected by centrifugation and washed in Buffer II (200mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM HEPES, pH 6.5). 200µl cell pellets were resuspended in 1ml of Lysis Buffer (0.5%SDS, 10mM EDTA, 50mM Tris, pH 8.1, 1x protease inhibitor cocktail (Roche) and 1mg/ml AEBSF) and sonicated 4 times for a 30 second interval of 0.5 second pulses (Fisher, Model 550 Sonic Dismembrator). Cell debis was removed by centrifugation, and the chromatin solutions were diluted 5x diluted with Dilution Buffer (1% Triton X-100, 2mM EDTA, 150mM NaCl, 20mM Tris, pH 8.1, 1x protease inhibitor cocktail). Chromatin fragments were immunoprecipitated with specific antibodies overnight at 4°C. For a 5-ml chromatin solution, the following amount of antibodies were used: 4.5 µg of α-AR, PG-21

(Upstate), 6 μg of α-CBP, A-22 (Santa Cruz), 15 μl of α-RNA polymerase II, 8WG16 (Covance), 10 μl of H5 (Covance), 12.5 μl of H14 (Covance), 3 μg of N-20 (Santa Cruz), 5 μl of α-AcH4 antiserum (Upstate), 7.5 μg of α-CDK7 C-4, α-CDK-8 C-19 and α-CDK-9 H-169 (Santa Cruz), 2 μg of α-HDAC1 (Upstate) or N-19 and α-HDAC2 C-19 (Santa Cruz), 22 μl of α-ACTR (Chen et al. 1999b), 30 μl of α-TIF2 rabbit antiserum raised against GST-TIF2 (aa800-1108). The ACTR and TIF2 antibodies do not cross-react in immunoprecipitation when tested with recombinant ACTR and TIF2 proteins (data not shown). Immunocomplexes were recovered and eluted as described before (Chen et al. 1999b). After reverse cross-linking at 65°C overnight, the DNA fragments were purified with QIAquick PCR Purification Kit (QIAGEN) and eluted with 100 μl of $TE_{1/10}$, pH 8.0. PCR was performed using 2-15 μl DNA and 26-28 cycles. Anti-polymerase II H5 and H14 immunoprecipitations were performed as above with the following modifications. 10 mM Na-pyrophosphate was added in the Lysis Buffer and Dilution Buffer. Protein A/G beads were pre-incubated with goat IgG α-mouse IgM overnight in Dilution Buffer and washed three times in Dilution Buffer before use. ChIP Re-IP was performed as described (Shang et al. 2000). Primers used in the ChIP assays are provided as Supplemental Materials. The ChIP results are representative of independent experiments performed at least three times.

The following primers were used for ChIP analysis of PSA and other gene sequences:

A: Forward: CAGTGTAATGCCATCCAGGGAAC
Backward: CGGTGTGATTTGTGCTGAAGG

B: Forward: AATCTAGCTGATATAGTGTGGCTC
Backward: AAGCATACACTTACACGGCACTCC

C: Forward: CTGCTGAATGCTTGGGATGTG
Backward: AAACAGGTGGGCACTGAGACTG

D: Forward: GTCCCCTCCTATCTCTATTCCCAG
Backward: CTAATCTCAGGTTTCTCACCAGTGG

E: Forward: CATGTTCACATTAGTACACCTTGCC
Backward: TCTCAGATCCAGGCTTGCTTACTGTC

F: Forward: ATTGCCTCCCAACGCTGTTC
Backward: CTCCCTCATTGACCTAAGAGTTCG

G: Forward: CAAACCCAGAATAAGGCAGCG
Backward: ATGATGAGTGGATGACACCCAAG

H: Forward: TCATCCACTCATCATCCAGCATC
Backward: GGAGAGCAATAGACTTGGGAAACC

I: Forward: TCTCCCAAGTGAGTCTCCCAGATAC
Backward: CCTCCACAGTGTCAATCCCAAAC

J: Forward: GCTGGGTTTGATTTTGGAGGTAG
Backward: AGCACAGTGAGGGAGACAGAAGTG

K: Forward: CACTTCTGTCTCCCTCACTGTGC
Backward: CCAGAGTAGCTCACTTGATCC

L: Forward: GGATCAAGTGAGCTACTCTGG
Backward: GTGGTGGACTACTGTAGTCTTAGC

M: Forward: CTCCTGCTTCGGTCTCCCAATAG
Backward: TGGCGCAATCTCGGCTCATTG

N: Forward: CCATGACTGACCTGTCGTTAATC
Backward: CCACAGATACATTGCTACTGTCATC

O: Forward: ACATTGTTTGCTGCACGTTGG
Backward: CAGGATGAAACAGAAACAGGGG

P: Forward: TCCTGAGTGCTGGTGTCTTAG
Backward: AGCCCTATAAAACCTTCATTCCCC

S: Forward: TTCCAGCAACTGAACCTCGC
Backward: GGGTTTGAAAAGACACGGGG

W: Forward: GGGAAGGAAAACAGGGTATGGG
Backward: AGAGAGAAGAACAAAACCAGGCAG

Z: Forward: AGATGGTCCTGGCCCTTGTC
Backward: ACTGCCCCATGACGTGATAC

U1: Forward: GTGAGGGGATCATGAGGTCAG
Backward: GGAACTATTCAAAAGAGTATACAGTACC

U2: Forward: GAATTTGGAGCTGGGATCTGAGG
Backward: GGAATGCATATTAAGGGTTGGTATGG

AR: Forward: GAACAGAGAGCCTCCCCAGAATC
Backward: CTCTCAAAGGCAAAATCACCCAGAC

Cyclin D1: Forward: TTCCTAGTTGTCCCCTACTGC
Backward: GACTCTGCTGCTCGCTGCTAC

CDK4: Forward: GTGGAGCGAAAAGGTGACAGCATC
Backward: CTGGGAGGAGGGCGAAGAGTGT

Transient Transfection

1 x 10^5 LNCaP cells were seeded in 24-well plates 24 hours before transfection in RPMI medium supplemented with 10% charcoal-stripped FBS. Cells were transfected with firefly luciferase reporter plasmids pGL3-basic (Promega), or its derivatives with PSA upstream sequences, pRL-SV40 Renilla luciferase plasmid (Promega), along with pCMX-ACTR or pSh-CMV-TIF2, using the Lipofectin reagent (Gibco BRL). Following transfection, cells were maintained in medium containing 1% charcoal-stripped FBS and treated with 10 nM DHT or 100 nM TSA (Sigma). Cells were harvested 24 hr after treatment, and the levels of luciferase were measured using a dual luciferase assay kit (Promega). The pGL3-PSA reporters were constructed by inserting restriction fragments of the PSA upstream regulatory sequences with the indicated lengths as follows, pGL3-PSA 630bp, EcoRI to HindIII; pGL3-PSA 5.8 kb, HindIII to HindIII; pGL3-PSA-E 1.5 kb, XbaI to BamHI; and pGL3-PSA-E-2.0 kb, XbaI to StuI.

RT-PCR

Gene expression was compared using RT-PCR. Total RNA was prepared with the TRIzol Reagent and the cDNA was synthesized with MMLV RT. PCR was performed using gene-specific primers (PSA_F: TGCCCACTGCATCAGGAACA, PSA_R: GTCCAGCGTCCAGCACACAG; β-$actin_F$: GAGAAAATCTGGCACCACACC, β-$actin_R$: ATACCCCTCGTAGATGGGCAC; AR_F: AAGAGCCGCTGAAGGGAAACAG,

AR_R: AGCATCCTGGAGTTGACATTGG; $CDK4_F$: AAGAGTGTGAGAGTCCCCAATGG, $CDK4_R$: GATTTTGCCCAACTGGTCGG; $CyclinD1_F$: TCCTGTGCTGCGAAGTGGAAAC, $CyclinD1_R$: AAATCGTGCGGGGTCATTGC). PSA and β-actin were amplified using 20 cycles and other genes were amplified with 26 cycles.

Recombinant adenovirus vector mediated gene expression

pShuttle-CMV vector (He et al. 1998) was modified to enhance the expression of ACTR and other long cDNA sequences (details of vector constructions is available upon request). Full-length or deletions of ACTR cDNA were inserted into the modified vector with 3 x HA tag sequence at the C-terminus. The resulting constructs were used to generate recombinant adenovirus following the protocols described previously (He et al. 1998). Viruses were purified by centrifugation in a CsCl step gradients. Viral titers were determined by OD_{260} reading and/or end-point cytopathic effect assay using 293 cells with GFP adeno-vector as a reference.

Co-immunoprecipitation and Western blotting

Approximately 5 x 10^6 LNCaP cells were infected with ACTR or GFP adenovirus at MOI 100 and then cultured in hormone-depleted medium for 48 hours before being treated with 1 nM synthetic androgen R1881 (NEN) for 2 hours. Cells were lysed at 4°C in buffer containing 50 mM Tris.HCl, 150 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM PMSF and 1x protease inhibitor cocktail and sonicated. Cell debris was removed by centrifugation. Cell lysates were pre-cleared by incubating with protein G agarose beads (Upstate) at 4°C for 1 hour and then incubated, at 4°C for 2 hours, with anti-HA antibody conjugated agarose beads (Santa Cruz). Immunoprecipitates were washed several times with cold PBS containing 0.2 % NP-40 and then eluted with 2 x SDS sample buffer. Samples were analyzed by Western blotting with specific antibodies.

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Figure legends

Figure 1. AR and p160-CBP/p300 coactivators are preferentially recruited to PSA enhancer to mediate androgen activation of PSA promoter in LNCaP cells.

(A) DHT induces the recruitment of AR and p160-CBP/p300 coactivators preferentially at the PSA enhancer. Panel a. A diagram of the 6-kb upstream regulatory region of AR target gene PSA with locations indicated for androgen-responsive enhancer and elements (ARE I and II), and the various fragments amplified in the ChIP assay marked as A to P (see Materials and Methods for details). Panel b. A portion of LNCaP cell genomic DNA prepared during the ChIP procedures (before incubation with antibody) were diluted, and the dilution at two different volumes (1x and 4x) was applied in the PCR reactions with different pairs of primers, but the same cycling conditions to monitor the relative amplification efficiency of the primers. Panels c-f. Indicated antibodies were used to perform ChIP assay with LNCaP cells treated with 10 nM DHT for 30 minutes or 4 hours, or mock treated (C) for 4 hours, to measure chromatin occupancy of AR (panel c), ACTR (panel d), TIF2 (panel e), and CBP (panel f) along the 6-kb sequence of PSA. Input corresponds to PCR reactions with DNA from 0.1% of total amount of chromatin solutions prepared from differentially treated LNCaP cells. **(B)** LNCaP cell transfection was performed to analyze the ability of PSA enhancer and/or promoter to mediate androgen transcriptional induction and coactivation function. LNCaP cells in hormone-

depleted media were cotransfected with either the 630-bp PSA proximal promoter-Luc reporter (a), or the 5.85-kb entire PSA upstream regulatory sequence-Luc (b), and the ACTR or TIF2 expression plasmids. Transfected cells were then treated with 10 nM DHT (filled bars), or mock treated (open bars), for 24 hours before measuring luciferase activities.

Figure 2. Androgen induces histone hyperacetylation at the PSA 5' regulatory region. LNCaP cells were either treated with 10 nM DHT or mock treated for 2 hours. ChIP assay with α–acetylated histone H4 antibody was used to measure the relative acetylation level at the 6 kb regulatory region. The precipitated DNA was analyzed by semi-quantitative PCR using primers from Fig 1A, plus two additional primer pairs amplifying regions centered at approximately –6,6 kb (U1) and –9,6 kb (U2). The relative histone acetylation levels (induction fold) at different chromatin fragments were determined by quantifying the PCR product using Flourochem® from Alpha-Innotech. DNA input before antibody addition from DHT or mock treated cells was determined to be the same (data not shown).

Figure 3. HDAC occupancy and function at PSA regulatory sequence in the absence of hormone or the presence of anti-androgen, and its displacement by DHT. (A) ChIP assay analysis of HDACs occupancy at PSA. LNCaP cells were maintained in

hormone-depleted media and treated with 10 nM DHT or 2 μM Casodex for indicated times. Cells were harvested for ChIP assay for the association of AR, HDAC1, HDAC2, and histone acetylation at the enhancer (fragments E and F) or the proximal promoter (fragments O and P) of the PSA gene. **(B)** Luciferase reporter assay analysis of transcriptional activation of PSA promoter by histone deacetylase inhibitor trichostatin A. LNCaP cells were transfected with either of the two reporters in Figure 1B, and then treated with 10 nM DHT, or 100 nM trichostatin A (TSA) for 24 hours prior to measuring luciferase activities.

Figure 4. RNA polymerase II is directly recruited to the PSA enhancer upon androgen stimulation of LNCaP cells. **(A)** ChIP assay was performed with α-PolII antibody (N-20) to analyze the PolII occupancy at the enhancer (fragment E, solid bars) or the promoter (fragment P, open bars) of the PSA gene, as in Fig B. **(B)** PolII occupancy over the entire PSA upstream regulatory sequence was analyzed using ChIP assay with monoclonal α-PolII antibody, 8WG16. **(C)** Two possible modes of androgen-induced PolII occupancy at the PSA 5' regulatory region: PolII is recruited to the enhancer and promoter independently (left), or PolII is recruited to the promoter and physically interacts with protein complex formed at the enhancer (right). **(D)** ChIP-ReChIP assay to analyze the interaction of Pol II with AR at the PSA promoter (O and P)

or the enhancer (E and F). LNCaP cells were treated with 10 nM DHT (+) or mock treated (-) for 1 hour prior to ChIP assay. Chromatin fragments were first immunoprecipitated with α-PolII (left) or α-AR antibody (right). Immunocomplexes were eluted from the agarose beads and diluted for a second immunoprecipitation to analyze for AR or PolII occupancy.

Figure 5. PolII recruited at PSA enhancer and promoter is phosphorylated at CTD distinctively. LNCaP cells were treated with 10 nM DHT for indicated times and ChIP assay was performed with PolII monoclonal antibodies: 8WG16 recognizes both unphosphorylated and partially phosphorylated PolII; H5 recognizes serine 2 phosphorylated PolII, and H14 recognizes serine 5 phosphorylated PolII. Co-precipitated DNA was analyzed for different forms of PolII occupancy at PSA enhancer (E and F), promoter (P), as well as the transcribed region (S, W and Z).

Figure 6. The PSA enhancer, in the isolation, mediates androgen induction of transcription independent of PSA proximal promoter. Luciferase reporter assay was performed as in Figure 1B with either the promoter-less pGL3 basic-Luc reporter, the 630-bp PSA proximal promoter-Luc reporter (PSA 0.6-kb), the 5.85-kb PSA upstream regulatory sequence-Luc reporter (PSA 5.8-kb), the Luc reporter with 1.5-kb fragment containing PSA enhancer (PSA-E 1.5-kb), or a similar reporter with 2.0-kb fragment

containing the enhancer (PSA-E 2.0-kb). Transfected cells were treated with 10 nM DHT (solid bars), or mock treated (open bars), for 24 hours before measuring luciferase activities.

Figure 7. CTD kinases are involved in PolII tracking from enhancer to promoter and androgen induction of PSA. **(A)** PolII CTD kinases are differentially recruited to PSA enhancer and promoter. Specific antibodies against Cdk7, Cdk8 and Cdk9 were used in ChIP assays with LNCaP cells treated with 10 nM DHT (DHT) or DHT plus 100 nM flavopiridol, a CTD kinase inhibitor (DHT+F). The recruitment of the three kinases to PSA enhancer and promoter was analyzed as in Fig 1. **(B)** RT-PCR was used to demonstrate that flavopiridol selectively blocks DHT induction of PSA expression. RNA was isolated from LNCaP cells treated with either 10 nM DHT (DHT) or DHT plus 100 nM flavopiridol (DHT+Flavo) for indicated times. **(C)** PolII tracking from enhancer to promoter is also blocked by flavopiridol. LNCaP cells were treated with DHT alone or DHT plus flavopiridol (DHT+F) as in part A. ChIP assay using α-PolII 8WG16, α-PolII H5, α–PolII H14 was performed to analyze the occupancy of the different phosphorylated forms of PolII at the enhancer and the promoter. **(D)** Flavopiridol does not affect PolII occupancy at the promoters of AR, CDK4 and Cyclin D1 genes. ChIP DNA from the same experiment as in (C) was analyzed for PolII occupancy at the promoters of indicated genes.

Figure 8. The p160 coactivator ACTR associates with PolII and P-TEFb, and mediates PSA transcription by increasing PolII recruitment at the PSA enhancer. **(A)** Adenovirus vector-mediated ectopic expression of ACTR in LNCaP cells. LNCaP cells were infected with adenovirus vector expressing GFP or HA-tagged ACTR full-length (WT-HA) or N-terminal truncated version of ACTR (A38-HA) at different MOIs (200 for GFP; 50, 100, and 200 for ACTR full-length and A38). Whole cell lysates were prepared 48 hours after infection and analyzed by Western blot with anti-HA and β-actin antibodies (top panel), or anti-ACTR monoclonal antibody (BD Biosciences, bottom panel), which recognizes both the endogenous and ectopically expressed full-length ACTR but not the N-terminal truncated ACTR-A38. **(B)** Ectopic expression of ACTR promotes androgen-dependent and –independent expression of PSA. RT-PCR was performed to measure PSA and β-actin expression in LNCaP cells infected with GFP-adenovirus or ACTR adenovirus vectors and treated with 1 nM DHT for indicated times prior to harvest. **(C)** Elevation of ACTR expression promotes PolII recruitment to the PSA enhancer. PolII occupancy at PSA enhancer and promoter was analyzed by ChIP assay as in Fig 7C using α-PolII 8WG16. LNCaP cells were harvested two days after infection with adenovirus vectors at MOI 100 for either GFP (open bars) or wild type ACTR (solid bars), and treated with 1 nM DHT for indicated times. Quantitation of the PCR products was made using Flourochem® from Alpha-Innotech. **(D)** Co-

immunoprecipitation assay to analyze the association of ACTR with AR, CBP, PolII and cyclin T1 in LNCaP cells. Whole cell lysates were prepared from LNCaP cells infected with ACTR-HA or GFP adenoviruses and treated with 1 nM synthetic androgen R1881. Lysates were immunoprecipitated with α-HA antibody conjugated agarose beads and Western blot analysis was used to determine the presence of endogenous AR and other proteins. Asterisk (*) indicates a non-specific polypeptide detected by the PolII antibody.

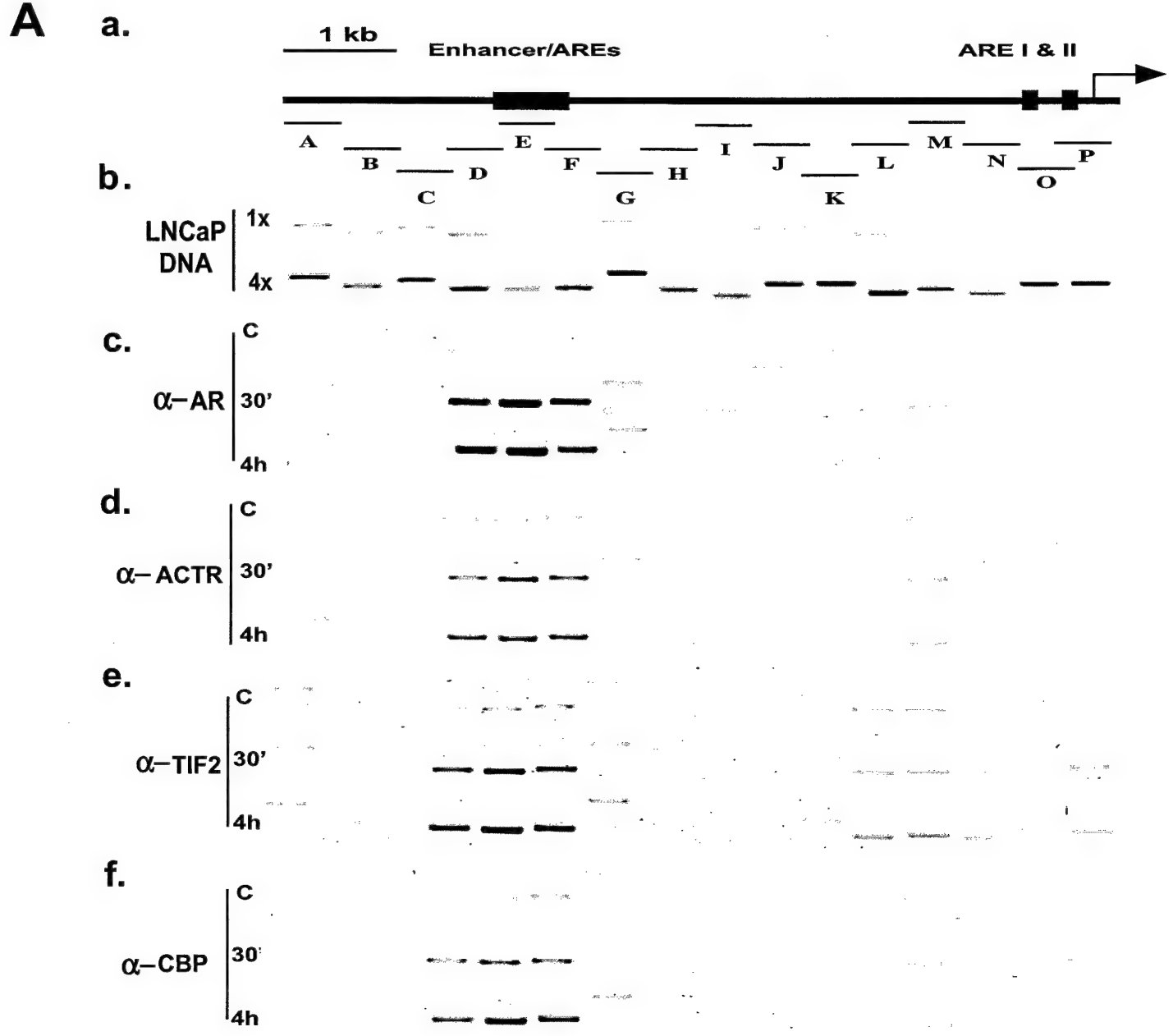
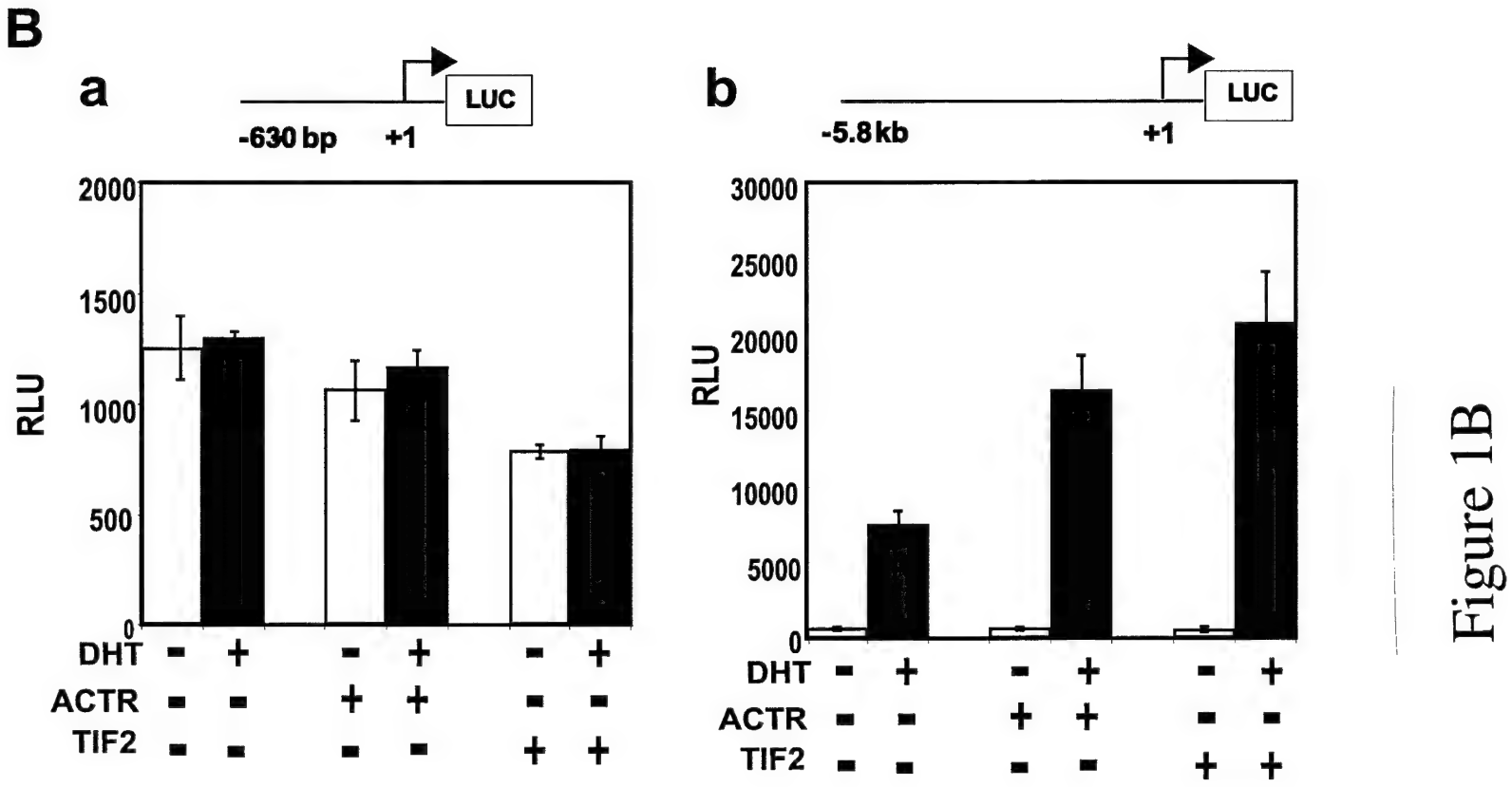

A
a.
1 kb
Enhancer/AREs
ARE I & II
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
b.
LNCaP DNA
1x
4x
c.
α–AR
C
30'
4h
d.
α–ACTR
C
30'
4h
e.
α–TIF2
C
30'
4h
f.
α–CBP
C
30'
4h

Figure 1A


B
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LUC
-630 bp
+1
2000
1500
1000
500
0
RLU
DHT - + - + - +
ACTR - - + + - -
TIF2 - - - - + +
b
LUC
-5.8 kb
+1
30000
25000
20000
15000
10000
5000
0
RLU
DHT - + - + - +
ACTR - - + + - -
TIF2 - - - - + +

Figure 1B

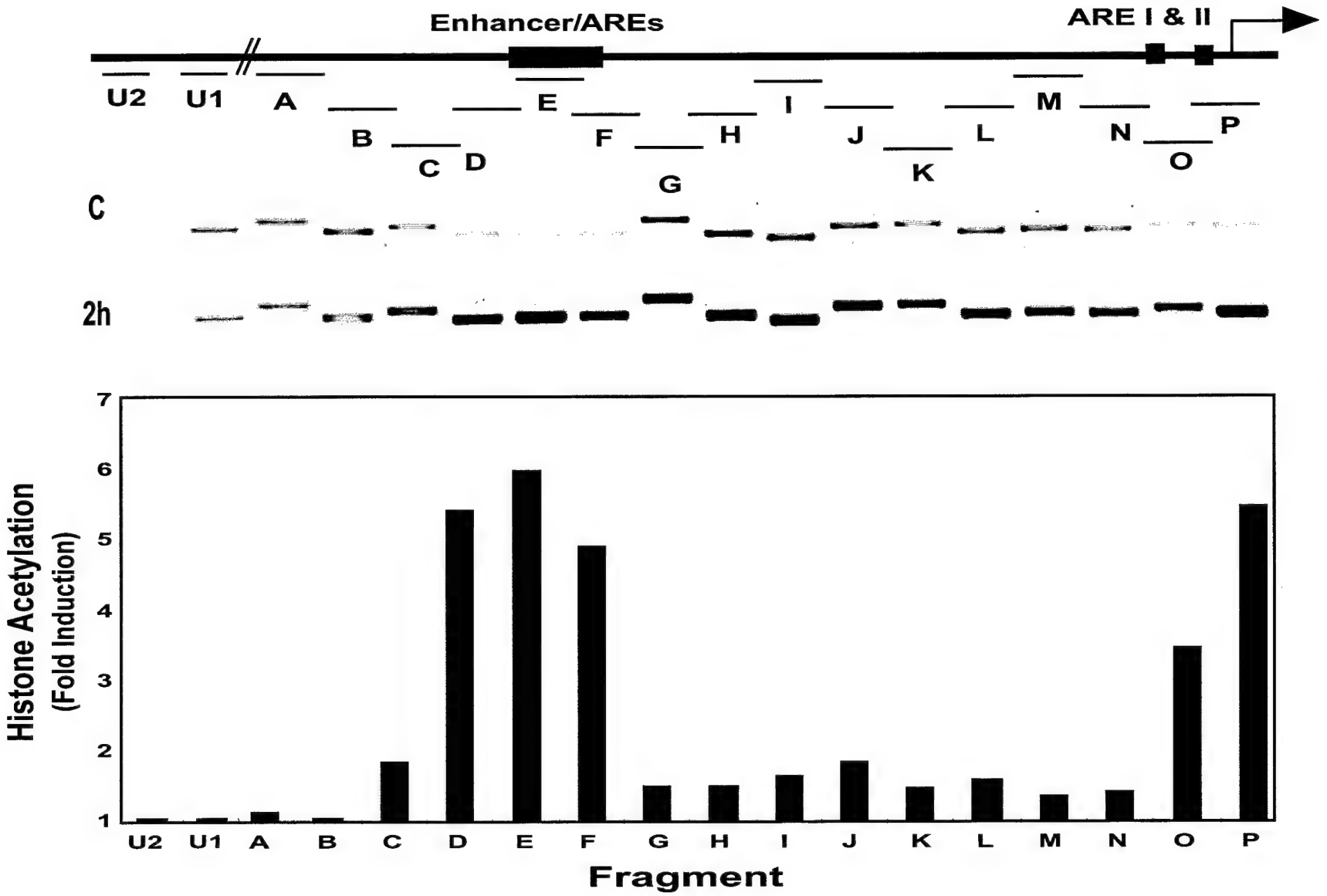

Enhancer/AREs
ARE I & II
U2
U1
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
C
2h
Histone Acetylation
(Fold Induction)
1
2
3
4
5
6
7
U2 U1 A B C D E F G H I J K L M N O P
Fragment

Figure 2

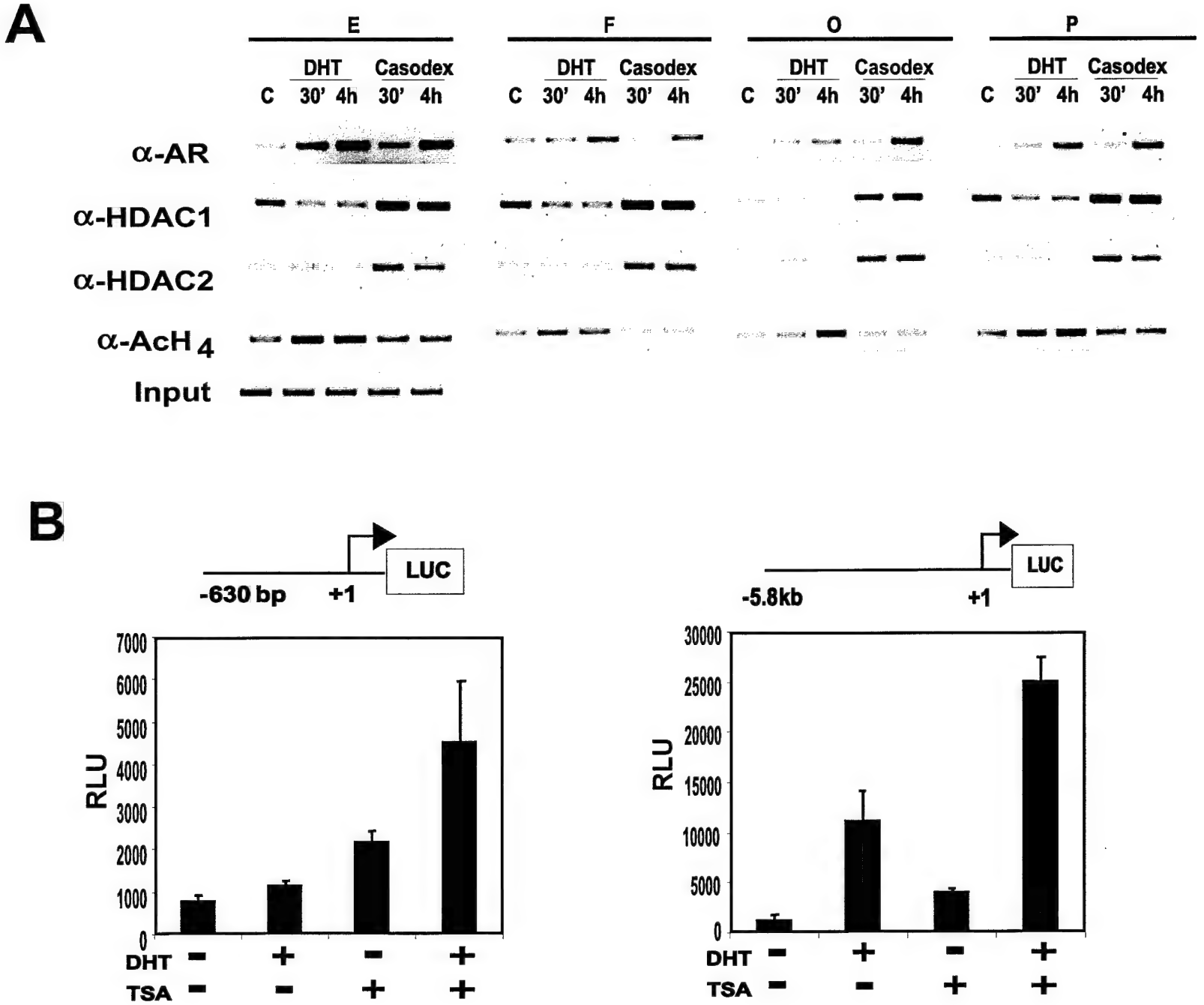

A
E
F
O
P
DHT
Casodex
C
30'
4h
α-AR
α-HDAC1
α-HDAC2
α-AcH4
Input
B
LUC
-630 bp
+1
-5.8kb
RLU
7000
6000
5000
4000
3000
2000
1000
0
30000
25000
20000
15000
10000
5000
DHT
TSA

Figure 3

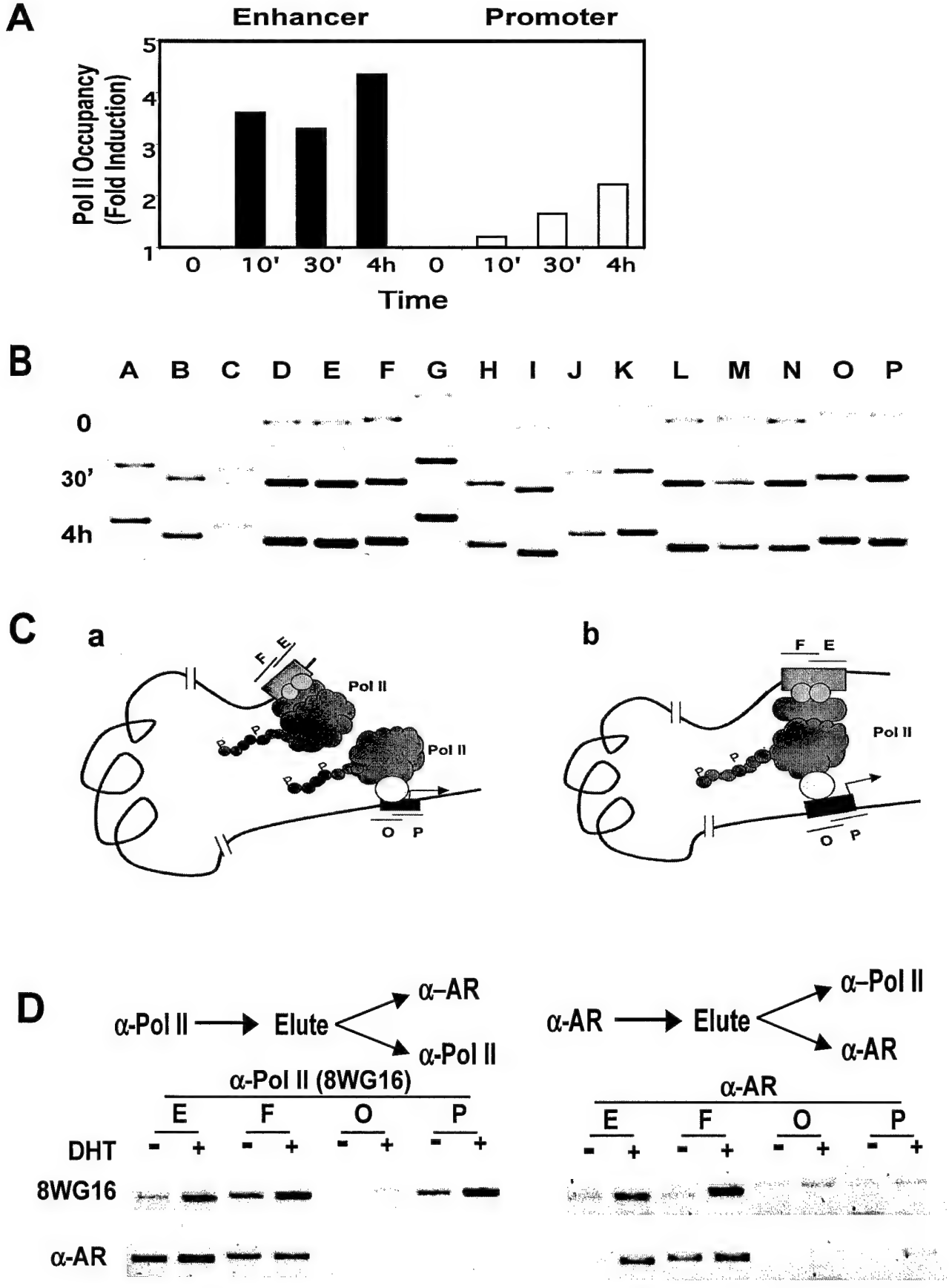

A
Enhancer
Promoter
Pol II Occupancy (Fold Induction)
5
4
3
2
1
0
10'
30'
4h
0
10'
30'
4h
Time
B
A B C D E F G H I J K L M N O P
0
30'
4h
C
a
F E
Pol II
Pol II
P P
P P
O P
b
F E
Pol II
P P
O P
D
α-Pol II
Elute
α–AR
α-Pol II
α-Pol II (8WG16)
E F O P
DHT
- + - + - + - +
8WG16
α-AR
α-AR
Elute
α–Pol II
α-AR
α-AR
E F O P
- + - + - + - +

Figure 4

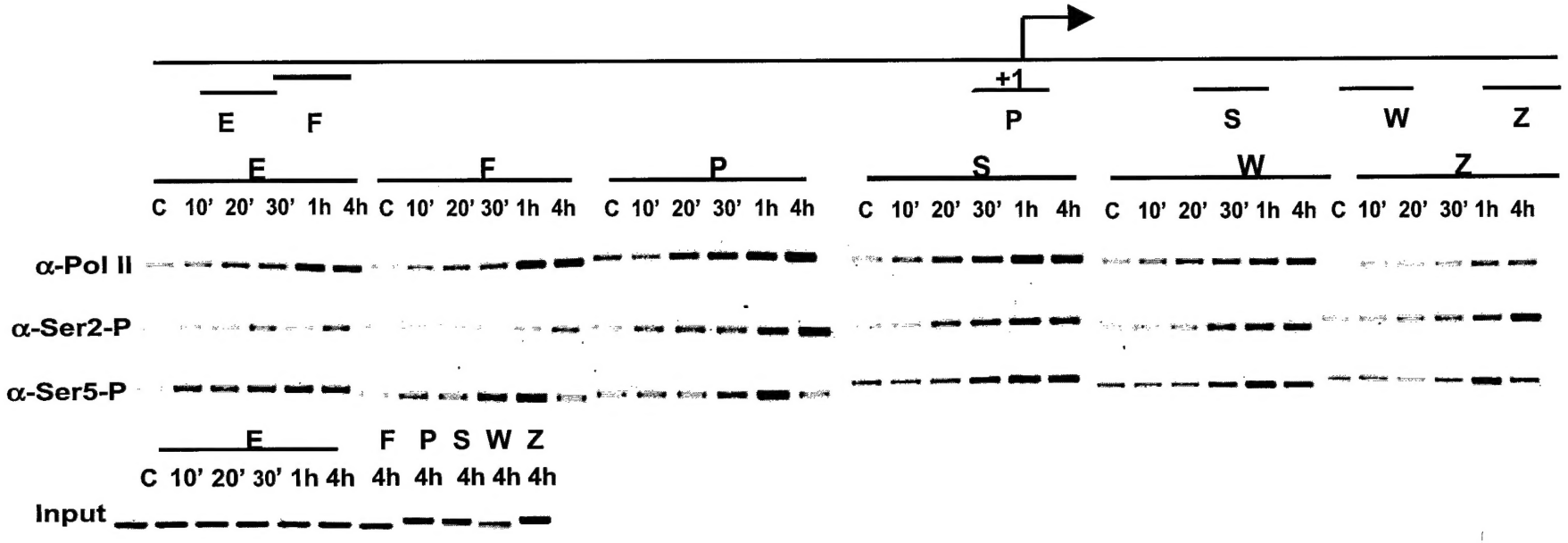
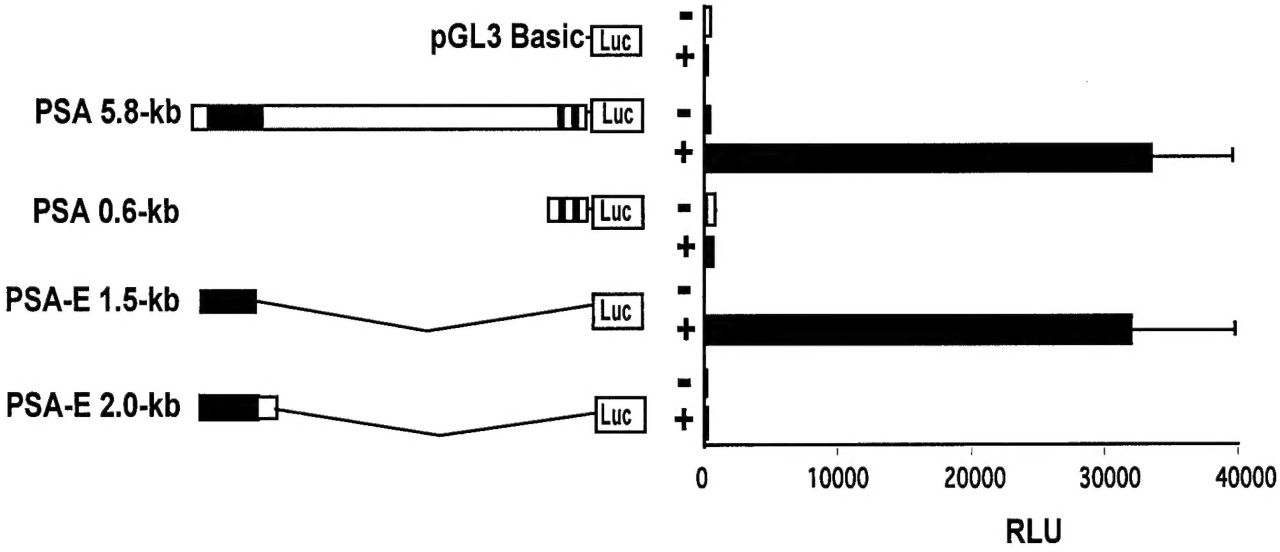

+1
E
F
P
S
W
Z
E
F
P
S
W
Z
C 10' 20' 30' 1h 4h C 10' 20' 30' 1h 4h C 10' 20' 30' 1h 4h C 10' 20' 30' 1h 4h C 10' 20' 30' 1h 4h C 10' 20' 30' 1h 4h
α-Pol II
α-Ser2-P
α-Ser5-P
E F P S W Z
C 10' 20' 30' 1h 4h 4h 4h 4h 4h 4h
Input

Figure 5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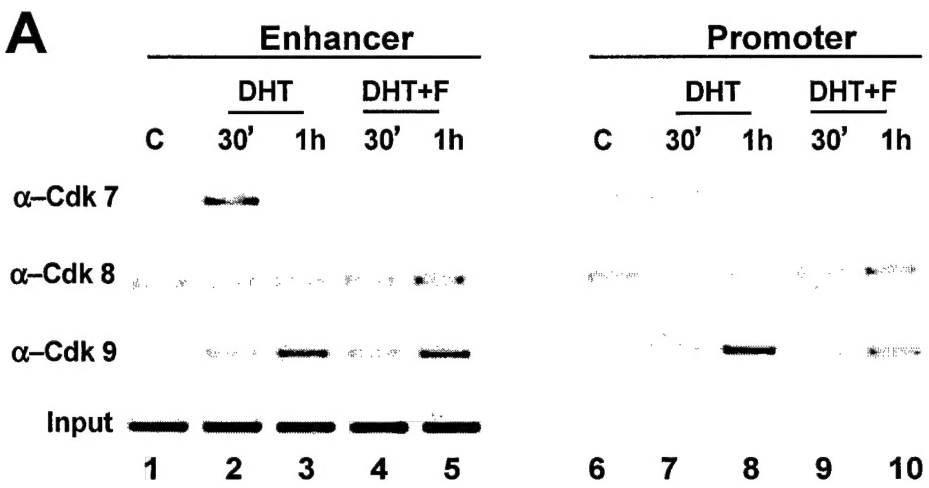
Luc
PSA 5.8-kb
Luc
PSA 0.6-kb
Luc
PSA-E 1.5-kb
Luc
PSA-E 2.0-kb
Luc
-
+
-
+
-
+
-
+
-
+
0
10000
20000
30000
40000
RLU

Figure 6


A
Enhancer
Promoter
DHT
DHT+F
C
30'
1h
30'
1h
α–Cdk 7
α–Cdk 8
α–Cdk 9
Input
1
2
3
4
5
6
7
8
9
10

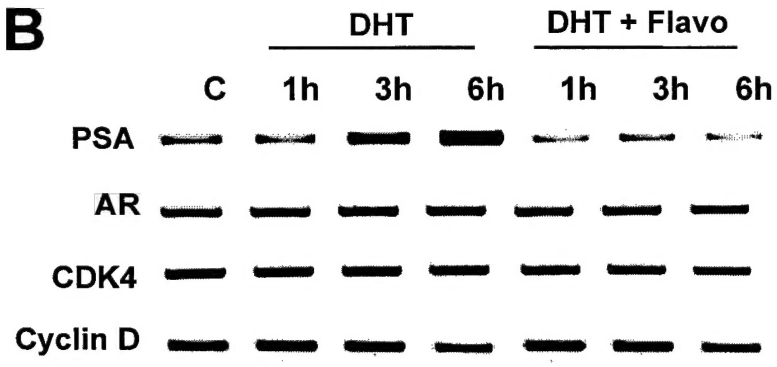
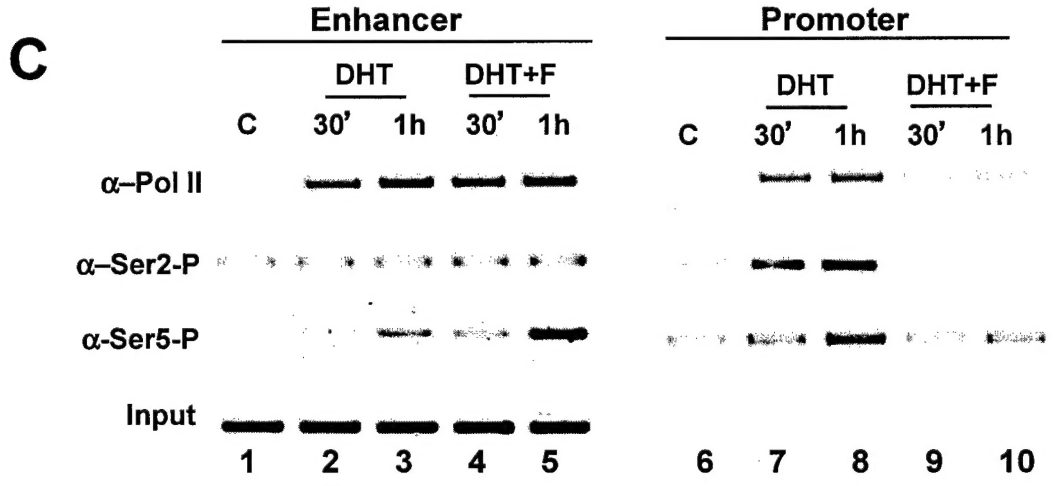

B
DHT
DHT + Flavo
C
1h
3h
6h
1h
3h
6h
PSA
AR
CDK4
Cyclin D

Figure 7


C
Enhancer
Promoter
DHT
DHT+F
C
30'
1h
30'
1h
α–Pol II
α–Ser2-P
α-Ser5-P
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1
2
3
4
5
6
7
8
9
10

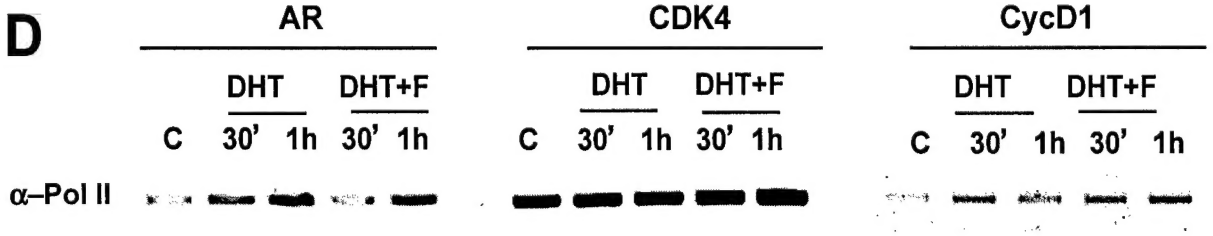
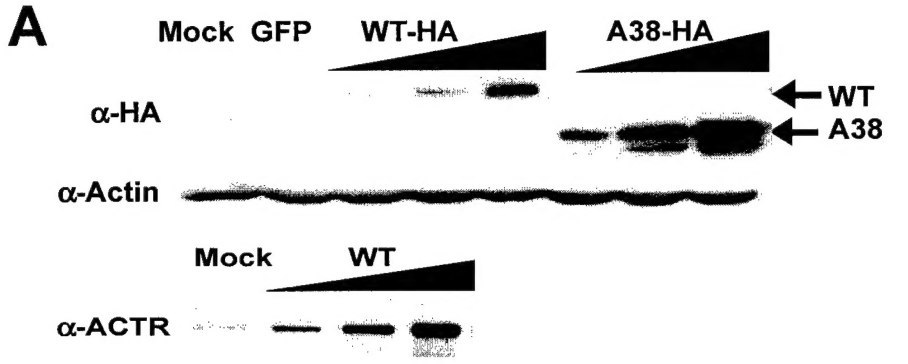

D
AR
CDK4
CycD1
DHT
DHT+F
C
30'
1h
30'
1h
α–Pol II

Figure 8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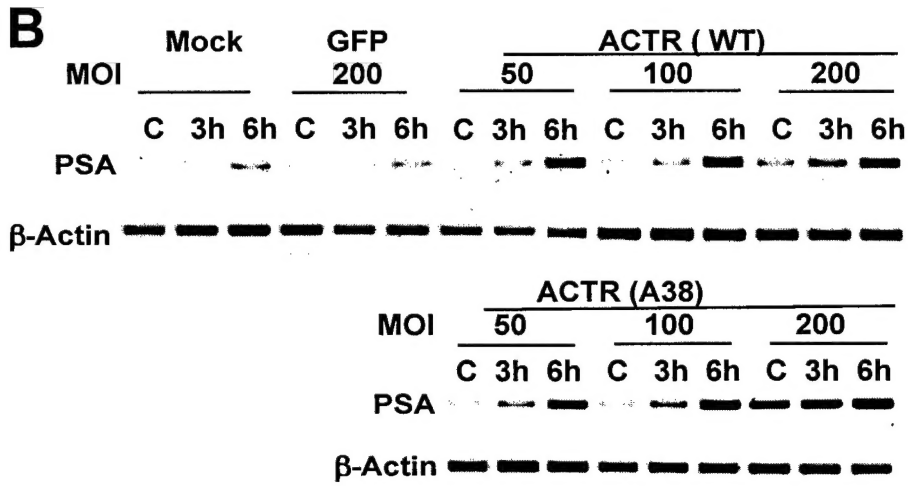
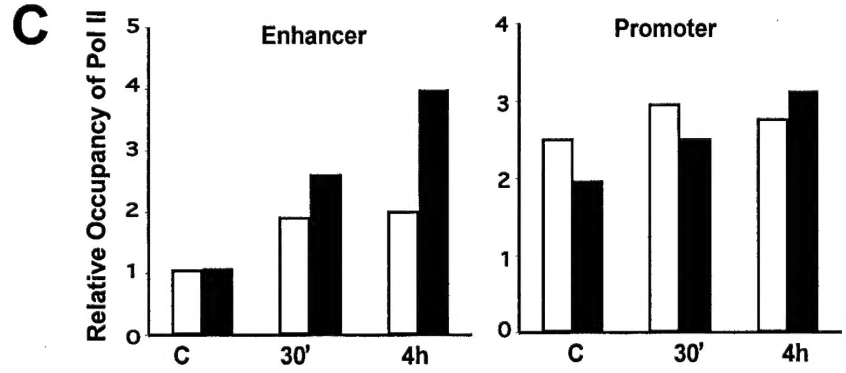
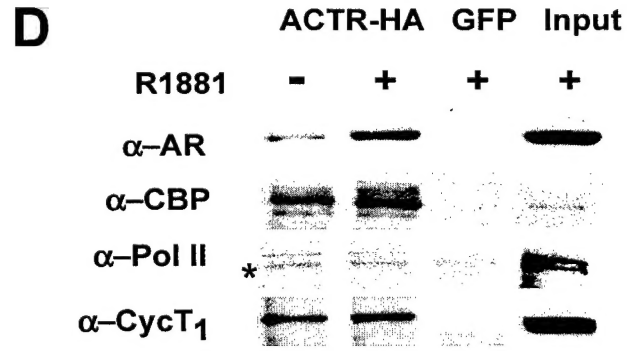
Mock GFP
WT-HA
A38-HA
WT
A38
α-HA
α-Actin
Mock
WT
α-ACTR


B
Mock
GFP
ACTR (WT)
MOI
200
50
100
200
C 3h 6h C 3h 6h C 3h 6h C 3h 6h C 3h 6h
PSA
β-Actin
ACTR (A38)
MOI
50
100
200
C 3h 6h C 3h 6h C 3h 6h
PSA
β-Actin


C
Relative Occupancy of Pol II
Enhancer
Promoter
0
1
2
3
4
5
C
30'
4h


D
ACTR-HA
GFP
Input
R1881
- + + +
α–AR
α–CBP
α–Pol II
*
α–CycT1